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# Importin-α3 Is Required at Multiple Stages of *Drosophila* Development and Has a Role in the Completion of Oogenesis

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**The *Drosophila importin-α3* gene was isolated through its interaction with the large subunit of the DNA polymerase α in a two-hybrid screen. The predicted protein sequence of Importin-α3 is 65–66% identical to those of the human and mouse importin-α3 and α4 and 42.7% identical to that of Importin-α2 (Oho31/Pendulin), the previously reported *Drosophila* homologue. Both Importin-α3 and Importin-α2 interact with similar subsets of proteins *in vitro*, one of which is Ketel, the importin-β homologue of *Drosophila*. *importin-α3* is an essential gene, whose encoded protein is expressed throughout development. During early embryogenesis, Importin-α3 accumulates at the nuclear membrane of cleavage nuclei, whereas after blastoderm formation it is characteristically found within the interphase nuclei. Nuclear localisation is seen in several tissues throughout subsequent development. During oogenesis its concentration within the nurse cell nuclei increases during stages 7–10, concomitant with a decline in levels in the oocyte nucleus. Mutation of *importin-α3* results in lethality throughout pupal development. Surviving females are sterile and show arrest of oogenesis at stages 7–10. Thus, Importin-α3-mediated nuclear transport is essential for completion of oogenesis and becomes limiting during pupal development. Since they have different expression patterns and subcellular localisation profiles, we suggest that the two importin-α homologues are not redundant in the context of normal *Drosophila* development.** 

***Key Words:*** **Importin-α; Karyopherin-α; nuclear transport; oogenesis; embryogenesis; *Drosophila*.**

## INTRODUCTION

Nucleocytoplasmic transport is a complex phenomenon, ensuring not only nuclear import of a variety of different proteins, but also the export of other proteins, tRNA, rRNA, mRNA, and ribosomal subunits into the cytoplasm (for review see Ullman *et al.*, 1997; Görlich, 1998; Mattaj and Englmeier, 1998; Stutz and Rosbach, 1998; Stochaj and Rother, 1999). The best-characterised nucleocytoplasmic transport mechanism is the nuclear localisation signal (NLS)-dependent nuclear protein import by means of an importin-α/β heterodimer. The basic-amino-acid-rich NLS plays a determinative role in the substrate specificity of this process (Koepp and Silver, 1996; Görlich, 1997; Dingwall and Laskey, 1998). Most of our knowledge of this system arises from *in vitro* nuclear import reconstitution experiments that make use of selectively permeabilized mammalian cells (Nigg, 1997; Görlich, 1998). These studies suggest that importin-α binds through its Armadillo (Arm) motifs to the NLS protein (Adam and Adam, 1994; Conti *et al.*, 1998) and through its importin-β binding domain (IBB) to importin-β (Moroianu *et al.*, 1995a; Görlich *et al.*, 1996; Weis *et al.*, 1996). Importin-β interacts with the nuclear pore complex (NPC) and the NLS protein/importin-α/importin-β trimeric complex is translocated into the nucleus in an energy-dependent manner (Görlich *et al.*, 1995; Moroianu *et al.*, 1995b). Inside the nucleus, in an environment with high RanGTP concentration, the direct binding of RanGTP to importin-β terminates the translocation of the trimeric complex, which then disassembles (Rexach and Blobel, 1995; Chi *et al.*, 1996; Görlich *et al.*, 1996). Consequently, the NLS protein is released from importin-α. Importin-β is probably exported to the cytoplasm as a complex

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with RanGTP and subsequently released from the complex by RanBP1 and RanGAP1 (Izaurralde *et al.,* 1997). The export of importin-$\alpha$ to the cytoplasm is mediated by the RanGTP/CAS/importin-$\alpha$ trimeric complex in which the CAS (cellular apoptosis susceptibility) protein interacts with NPC (Kutay *et al.,* 1997; Herold *et al.,* 1998). In the cytoplasm RanBP1 causes the dissociation of RanGTP from the RanGTP/CAS/importin-$\alpha$ complex, and reassociation is prevented by RanGAP1-triggered GTP hydrolysis (Bischoff and Görlich, 1997). The RanGTP-free CAS is now in a low-affinity form for importin-$\alpha$ binding and importin-$\alpha$ is released. In the cytoplasm, where the RanGTP concentration is low, the importin-$\alpha$ and $\beta$ will combine to reform the nuclear import-competent heterodimer. Despite the functional characterisation of these key components, several aspects of NLS-protein import are still poorly understood. It is not clear for example whether the multiple isoforms of importin $\alpha$ and $\beta$ present in many species have specific functions or whether they are functionally redundant. However, the high degree of identity observed in the case of importin-$\alpha$ and $\beta$ proteins in different model organisms suggests the evolutionarily conserved nature of the NLS-protein import.

*Drosophila* genes encoding importin-$\alpha$ and $\beta$ homologues have already been reported. The *oho31/pendulin* gene encodes an importin-$\alpha$2 subfamily member, which is present at high levels in early embryos and is rapidly degraded at the end of embryogenesis (Kussel and Frasch, 1995; Török *et al.,* 1995). This protein shows cell cycle-dependent nuclear localisation, accumulating in the nucleus at the prophase of embryonic divisions. The *ketel* gene encodes an importin-$\beta$ homologue required for viability throughout development (Erdélyi *et al.,* 1997).

In this study, we report the isolation of the *importin-α3* (*imp-α3*) gene of *Drosophila* through its interaction with the NLS-containing protein DNA polymerase $\alpha$ in a yeast two-hybrid screen. The predicted sequence of Importin-$\alpha$3 shows the characteristic IBB domain, Arm motifs, and C-terminal acidic region. Importin-$\alpha$3 protein is expressed at almost constant levels throughout *Drosophila* development and during interphase accumulates in nuclei, while in M-phase is cytoplasmic. The *imp-α3* mutant exhibits lethality in late larval to pharate adult stages, underlining the importance of nuclear transport during development. A female sterile phenotype characterised by the degenerative ovaries of the *imp-α3* mutants also suggests a role for this importin-$\alpha$ during oogenesis. The subcellular localisation of Importin-$\alpha$3 is discussed in the light of the *Drosophila* development and compared to the previously reported Importin-$\alpha$2.

## MATERIALS AND METHODS

### Fly Strains and Culture Conditions

Wild-type and mutant strains were maintained and mated on standard yeast–agar–cornmeal medium and all experiments were performed at 25°C. All genetic markers and mutations used are described in Lindsley and Zimm (1992). Deficiency stocks *Df(3R)by10* and *Df(3R)by62* were obtained from the Umea Stock Center. The *imp-α3*$^1$ line was originally designated *335/13* (Deák *et al.,* 1997). The *TM3, Sb, ry, [Δ2-3, ry*$^+$*]/Df(3R)C7, ry*$^{507}$ stock was given by János Gausz. All the stocks utilised were isogenised and put on a $w^{1118}$ background.

### Mutant Phenotype Determination

The homozygous and hemizygous *imp-α3*$^1$-associated mutant phenotypes were determined on a *TM6b, Tb* or a *TM6c, Tb, Sb* background as described by Deák *et al.* (1997). Individual sterility tests were performed for homozygous and hemizygous *imp-α3*$^1$ females by crossing them to wild-type Canton S males.

### Remobilization of the P-lacW Element

In order to revert the *imp-α3*$^1$ mutation the P-lacW element was remobilised under dysgenic conditions. About 250 jump-starter males of genotype $w^{1118}$*/Y; imp-α3*$^1$*/TM3, Sb, ry, [Δ2-3, ry*$^+$*]* were crossed individually to $w^{1118}/w^{1118}$*; TM3, Sb, Ser/TM6b, Tb* virgins. From their progeny the *imp-α3*$^1$*/TM3* and *imp-α3*$^1$*/TM6b* flies were scored for $w^-$ or modified $w^+$ expression compared to original $w^+$ expression level seen in the eyes of *imp-α3*$^1$ flies. For each jump-starter male, only one fly was selected showing the $w^-$ or the modified $w^+$ phenotype and for these revertant strains were established over *TM6b, Tb*.

### Cloning and Sequencing

Yeast two-hybrid screening was carried out as described (Ruden *et al.,* 1991; Gyuris *et al.,* 1993; Zervos *et al.,* 1993). Roger Brent kindly provided the yeast strain EGY48, the lacZ reporter plasmid (pSH18-34), the LexA fusion control plasmid (RFHM1), and the plasmid library (*Drosophila* ovarian cDNA in pJG4-5–RFLY3). The plasmid used to make the LexA fusion bait was based on pRS313 and pV44ER-LEX. The *Sac*I–*Kpn*I fragment of pV44ER-LEX, containing the galactose-inducible LexA protein and a multiple cloning site, was ligated into *Sac*I–*Kpn*I-cut pRS313 in order to change the selectable marker from Trp to His. The *Drosophila* DNA polymerase-$\alpha$ p180 subunit-coding region was then cloned in frame with the LexA protein.

DNA sequencing was carried out using the Sequenase kit (US Biochemicals, Cleveland, OH) or was sent to Advanced Biotechnology Centre, Charing Cross and Westminster Medical School, for ABI sequencing. Nucleotide sequence analysis and amino acid comparisons were performed using Geneworks (IntelliGenetics, Inc.) for the Macintosh and the BLAST programs at NIH. The 5′ region of *imp-α3* was amplified by PCR from the RFLY3 library using one primer from the pJG4-5 vector, 5′-GCCTC-CTACCCTTATGATGTGCCAG-3′, and another, 5′-GAATGGT-GGCTGTCGGGGGAGCCGGATCCTT-3′, from within the *imp-α3* coding region. The *Drosophila* cDNA clones GM01016, GM05410, and GM06753 were obtained from Genome Systems, Inc., and they were sequenced in the Department of Genetics, University of Cambridge.

The P-lacW-mediated plasmid rescue and the salivary gland polytene chromosome *in situ* hybridisation were carried out as described by Deák *et al.* (1997).

Both Southern and Northern blots were carried out according to standard procedures (Sambrook *et al.,* 1989). For Southerns, genomic DNA was extracted from wild-type and mutant *imp-α3*

lines and then restriction digestions were performed. Poly(A)$^+$ RNA from all developmental stages of *Drosophila* was purified using the Oligotex mRNA kit from QIAgen.

### *Drosophila Germ-Line Transformation*

A 9-kb genomic fragment containing the entire *imp-α3* gene was cloned into pCasper4 and the resulting pR4 construct together with the transposase encoding the pII25.7WC plasmid were injected into $w^{1118}$ flies. Six independent transgenic lines were obtained and among them the $P[imp\text{-}\alpha3^+]^{42}$ homozygous viable, second-chromosome insertion line was used to rescue the *imp-α3*-associated mutant phenotypes. A defective construct, pRD4.1, was created by deleting a *Bsi*WI–*Swa*I fragment (Fig. 1). Following germ-line transformation one $P[imp\text{-}\alpha3^-]^{411}$ homozygous viable, first chromosome insertion line was established.

### *Production of Importin-α3 Antibody*

The region of *imp-α3* coding for amino acids 227–514 was cloned into pQE31 (QIAgen) and the His-tagged protein expressed and purified according to the manufacturer's manual. An amount of 0.8 mg of protein was sent to Neosystem Laboratoire for antibody production in two rabbits. The polyclonal antiserum of these two rabbits was affinity purified and used on Western blots.

### *Preparation of Drosophila Protein Extracts*

To make cytoplasmic and nuclear extracts, dechorionated wild-type *Drosophila* embryos were homogenised in the presence of protease inhibitors, with a loose pestle to maintain the integrity of the nuclei. The homogenate was filtered through two layers of Miracloth to remove cell debris and centrifuged at 25K for 20 min in a Beckman TL100. The resulting supernatant was the cytoplasmic fraction. The nuclear pellet was washed five times in TP3 buffer (10 mM Hepes, pH 7.9, 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, protease inhibitors). Nuclei were broken open by adding an equal volume of Buffer A (50 mM Hepes, pH 7.9, 50 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM EDTA) and mixing at 4°C for 30 min. This was repeated to give fractions T1 and T2. Proteins bound to nuclei were released with low-salt (Buffer A + 50 mM NaCl) followed by high-salt (Buffer A + 2 M NaCl) extractions. The remaining pellet was sonicated.

Total extracts for immunoprecipitations and GST pull-downs were prepared in a similar manner except that homogenisation was carried out in IP buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 0.1% Tween 20, protease inhibitors) at 1 g/ml embryos, with a loose followed by a tight pestle to break open nuclei. After filtration through Miracloth, nuclear debris was removed by centrifugation at 4°C at low speed (6.5 K) in a bench-top microcentrifuge. The supernatant was either used immediately or mixed with glycerol at 10% and frozen as droplets in liquid nitrogen.

### *Developmental Western Blot Analysis*

Total protein extracts corresponding to the different developmental stages of *Drosophila* were made into standard 2× SDS protein loading buffer. Equal amounts of protein samples were loaded onto 10% polyacrylamide gels and after electrophoresis electroblotted onto Hybond ECL (Amersham) or Immobilon-P (Millipore) membranes. α-Tubulin was used as a loading standard using monoclonal α-tubulin antibody (Amersham). The peroxidase-labelled anti-rabbit and anti-mouse secondary antibodies were purchased from Amersham or Jackson Immunoresearch Laboratories, Inc. The blots were developed using ECL kit (Amersham) or SuperSignal Substrate Western Blotting (Pierce). Quantitation of ECL blots was carried out by comparing the sample to titrated amounts of overproduced protein on the same blot. Total ovarian protein extracts were prepared from 15 either wild-type or $imp\text{-}\alpha3^1/imp\text{-}\alpha3^1$ females. Equal volumes of ovarian protein samples were analysed on Western blots as described above.

### *Western Analysis of Drosophila Cell Extracts*

Full-length open reading frames of *imp-α3* and *oho31* were cloned in frame into pGEX2TK. Proteins were induced, purified, and labelled with [$\gamma$-$^{32}$P]ATP according to manufacturer instructions. Total *Drosophila* extracts (cytoplasmic, high-salt nuclear wash, and nuclear pellet) were electrophoresed on 12% SDS–PAGE and blotted onto nitrocellulose. Blots were incubated overnight in BB buffer (20 mM Hepes, pH 7, 10% glycerol, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20) plus 5% milk powder at 4°C to block and also to allow the proteins to renature. Probes were incubated with the renatured blots for 1 h at 4°C, washed five times with BB buffer, and exposed to film.

### *GST Pull-downs*

One millilitre of 0- to 5-h embryo extracts (see above) was incubated at 4°C for 1 h with an equal amount of GST or GST–Importin-α1 or GST–Importin-α3 fusion protein bound to glutathione agarose (Pharmacia). The beads were then washed extensively in IP buffer before elution of bound proteins with IP buffer containing 100, 200, and 500 mM NaCl. Proteins eluted in high salt were directly visualised by silver staining.

### *Immunoprecipitations*

Antibodies were crosslinked to protein A–Sepharose beads using dimethylpimelimidate. An amount of 40 μl crosslinked beads was incubated for 1 h at 4°C with 200 μl *Drosophila* 0- to 4-h whole-embryo extracts. Beads were then washed 10 times with IP buffer plus 100 mM NaCl (1 ml per wash) and resuspended directly into 40 μl 2× SDS–PAGE loading buffer.

### *Immunocytochemistry and Confocal Microscopy*

Cytological preparations of third-instar larval brains were made according to Sunkel and Glover (1988). For each genotype 10 microscopic fields per brain from 10 brains were analysed, using the phase-contrast Neofluar 60× oil Nikon objective, the 10× oculars, and the Optovar set at 1.25×.

Ovaries were dissected in 2× PBT (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $K_2HPO_4$, 0.2% Triton X-100, pH 7.5) and fixed with 5% formaldehyde or paraformaldehyde in PBS for 20 min. A second fixation was carried out in absolute methanol for 30 min and then the samples were rehydrated in 2× PBT for 1 h. Overnight incubations were done with the primary antibodies at 4°C and the incubations in the secondary antibodies lasted for 4 h at room temperature. The fixation and immunostaining of embryos were performed according to Máthé *et al.* (1998) or Maldonado-Codina and Glover (1992). The Importin-α2 was detected with the rabbit anti-Pendulin antibody. The microtubules were detected with YL1/2 rat monoclonal anti-α-tubulin antibody (Sera Lab, Inc.),

while the T47 mouse monoclonal anti-lamin antibody was used to visualise the nuclear lamina (Paddy *et al.*, 1990). The Alexa 488 anti-rat or anti-mouse and Texas red anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch Laboratories, Inc. Digital images of optical sections were collected with a Bio-Rad 1024 confocal microscope.

## RESULTS

### *Structural Characteristics of the Importin-α3 Protein*

Two proteins showing significant homology to the importin-α protein family were identified by a yeast two-hybrid screen as proteins that interact with the large subunit of DNA polymerase α. One of these had been previously reported as Oho31/Pendulin (Kussel and Frasch, 1995; Török *et al.*, 1995), which we will refer to as Importin-α2 based on its homology to the importin-α2 subfamily. The second is a new importin-α homologue of *Drosophila*, which we named Importin-α3, similarly based on its homology to the importin-α3 protein subfamily—see below. Both of these also showed interactions with DNA polymerase α in immunoprecipitates from crude yeast extracts (data not shown).

The original cDNA clone of *imp-α3* isolated from the yeast two-hybrid screen was found to be truncated at the N-terminus, starting 622 bases into the coding region. We therefore used PCR to isolate the upstream coding region from the same cDNA library used for the initial two-hybrid screen. A clone of 2.2 kb containing the full-length cDNA was then constructed by splicing together the upstream and downstream regions by means of a unique *Bam*HI site in the primer region (Fig. 1). The EMBL Database Library accession number of the *imp-α3* cDNA and promoter region is AJ237997. BLAST searches with this cDNA sequence revealed three *Drosophila* ESTs, and the corresponding cDNA clones GM01016, GM05410, and GM06753 (Genome Systems, Inc.) were actually identical to our full-length cDNA (GenBank Accession Nos. AF230871, AF230872, and AF230873).

Analysis of the nucleotide sequence of the 2.2-kb cDNA clone showed that there are two possible start codons very close together, but only the first is immediately preceded by the *Drosophila* translation start consensus sequence ACAAA (Cavener, 1987). Moreover, according to Kozak (1995), when there are two ATG start codons very close together the first codon is usually the one at which initiation occurs. We therefore chose to assign the open reading frame from the first ATG to the TAA stop codons such that it encoded a protein of 514 amino acids. The open reading frame is followed by a 524-nucleotide-long untranslated region containing several close, but not identical, matches to the AATAAAA poly(A)-addition signal before the start of the poly(A) tract. The calculated molecular mass of Importin-α3 is 57 kDa, which is in agreement with our subsequent Western blot analysis (Fig. 4). The most highly conserved central region of the Importin-α3 is predominantly hydrophobic and is composed of eight Arm motifs. The N- and C-termini are both hydrophilic and contain previously characterised motifs. The N-terminus contains a stretch of amino acids (residues 6–45) which is an absolute match for the IBB domain, shown in the case of other importin-α homologues to be essential for importin-β binding and nuclear import (Weis *et al.*, 1996; Görlich *et al.*, 1996; Conti *et al.*, 1998) (Figs. 2A and 2B). The C-terminus has a strongly acidic amino acid stretch (residues 430–514), which was shown for the human importin-α2 to be involved in CAS binding (Herold *et al.*, 1998). Multiple alignment analysis of Importin-α3 with other members of the importin-α protein family revealed that its closest relatives (65–66% identity) are the mouse and human importin-α3 and α4 homologues (the mouse homologues were also described as importin α-Q1 and α-Q2; Tsuji *et al.*, 1997) (Fig. 2C). This is a higher homology than that observed between Importin-α3 and the other reported *Drosophila* homologue Importin-α2 (42.7%). The *Drosophila* Importin-α2 is in turn more closely related to members of the importin-α2/Rch1/α-P1 subfamily from other species.

### *Importin-α3 and Importin-α2 Interact with a Similar Subset of Proteins in Vitro*

To compare proteins interacting with Importin-α3 and Importin-α2 we prepared nuclear and cytoplasmic protein fractions of a *Drosophila* embryonic extract. The protein fractions were separated by electrophoresis, blotted, and then probed with $^{32}$P-labelled full-length GST fusion proteins (Fig. 3A). Importin-α3 and Importin-α2 recognise similar patterns of interacting proteins in all types of extract, whereas GST alone showed no interactions. A greater number of interacting proteins were detected in the high-salt nuclear wash fraction compared with the pellet and the cytoplasmic fraction, which showed only one protein of approximately 50 kDa. Since some proteins may not renature correctly during the electroblotting procedure we also looked for proteins within embryonic extracts that would interact directly with GST–Importin-α2 or GST–Importin-α3 immobilised on glutathione beads (Fig. 3B). After the beads were washed with 200 mM NaCl, the bound proteins were eluted with 500 mM NaCl. From Fig. 3B it can be again seen that similar sets of proteins interact with GST–Importin-α2 and GST–Importin-α3, but not with the control GST alone, the most prominent being of 90, 70, and 50 kDa. The 90-kDa band corresponds to Ketel, the *Drosophila* homologue of the importin-β protein, as shown by Western blotting of the eluted proteins with antibodies against the Ketel protein (a kind gift from János Szabad). The identity of the other two bands has not been determined.

Immunoprecipitations from 0- to 5-h embryonic extracts using anti-Importin-α3 antibodies also revealed interactions with proteins of 90 and 70 kDa (data not shown). The interaction of the 50-kDa protein could not, however, be

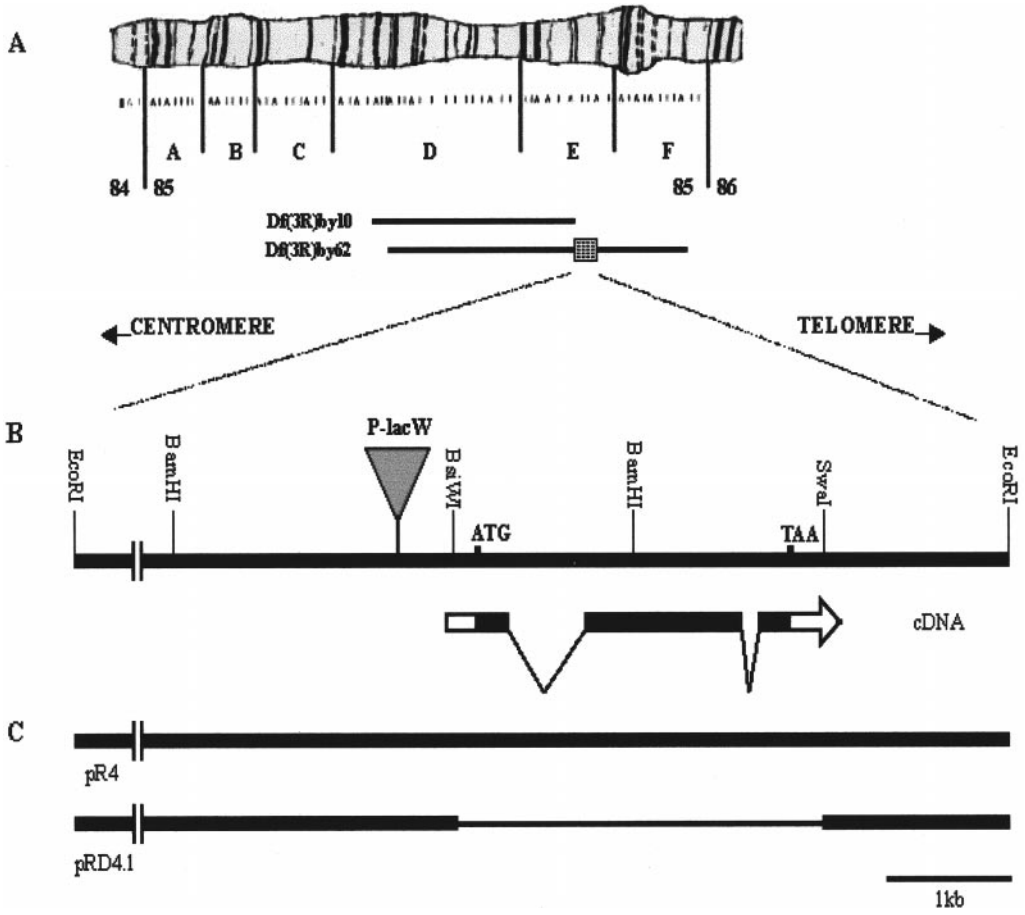


**FIG. 1.** Genetic and molecular dissection of the *imp-α3* region. (A) The *imp-α3* locus maps by *in situ* hybridization to polytene chromosomes to the 85E10–13 region of the third chromosome (stippled box) and is defined by the chromosome deficiency *Df(3R)by62* and a P-lacW insertion. (B) Restriction map of a 9-kb *Eco*RI genomic fragment, showing the P-lacW insertion in the 5′ region of the *imp-α3* gene. The genomic region between the P-lacW and the distal *Eco*RI site contains a single transcription unit of 2.2 kb. The corresponding cDNA clone is shown beneath the genomic fragment. The open boxes represent the noncoding transcribed regions and the filled boxes stand for the open reading frame. (C) The *imp-α3* rescue constructs. The pR4 construct contains the 9-kb *Eco*RI genomic fragment and rescues the homozygous and hemizygous $imp\text{-}\alpha3^{1}$ mutant phenotypes. The pRD4.1 defective construct is internally deleted (*Bsi*WI–*Swa*I fragment) within the *imp-α3* gene and cannot rescue the mutant phenotypes.

confirmed by this method due to the masking of this region of the gel by bands from the antibody. Although we cannot role out differences in the strengths of the interaction for each protein, it seems that Importin-α2 and Importin-α3 interact with similar sets of proteins *in vitro* that might correspond to other members of the NLS-transport machinery yet unidentified in *Drosophila.*

### *Importin-α3 Is Present throughout Development*

Probes from either the full-length cDNA or the genomic region of *imp-α3* detect a single transcript of 2.2 kb that was present in poly(A)$^+$ RNA throughout development, being particularly abundant in female flies and early embryos (data not shown). To define the developmental profile of Importin-α3 protein expression, we raised antibodies against the C-terminal portion of Importin-α3 and used these to probe Western blots containing total protein extracts from a range of developmental stages (Figs. 4A and 4B). A single band of 58 kDa, which was present at a relatively constant level throughout development and elevated in adult females, was recognised. A more detailed comparative Western analysis shows Importin-α3 present at a very low level in 0- to 2-h embryos and increasing in amount in 2- to 4-h embryos (Fig. 4A). In contrast, Importin-α2 is found at a very high level in the early embryo and then shows a rapid decrease in abundance during late embryogenesis, while in pupae and adult females its expression level is significantly reduced (Fig. 4D).

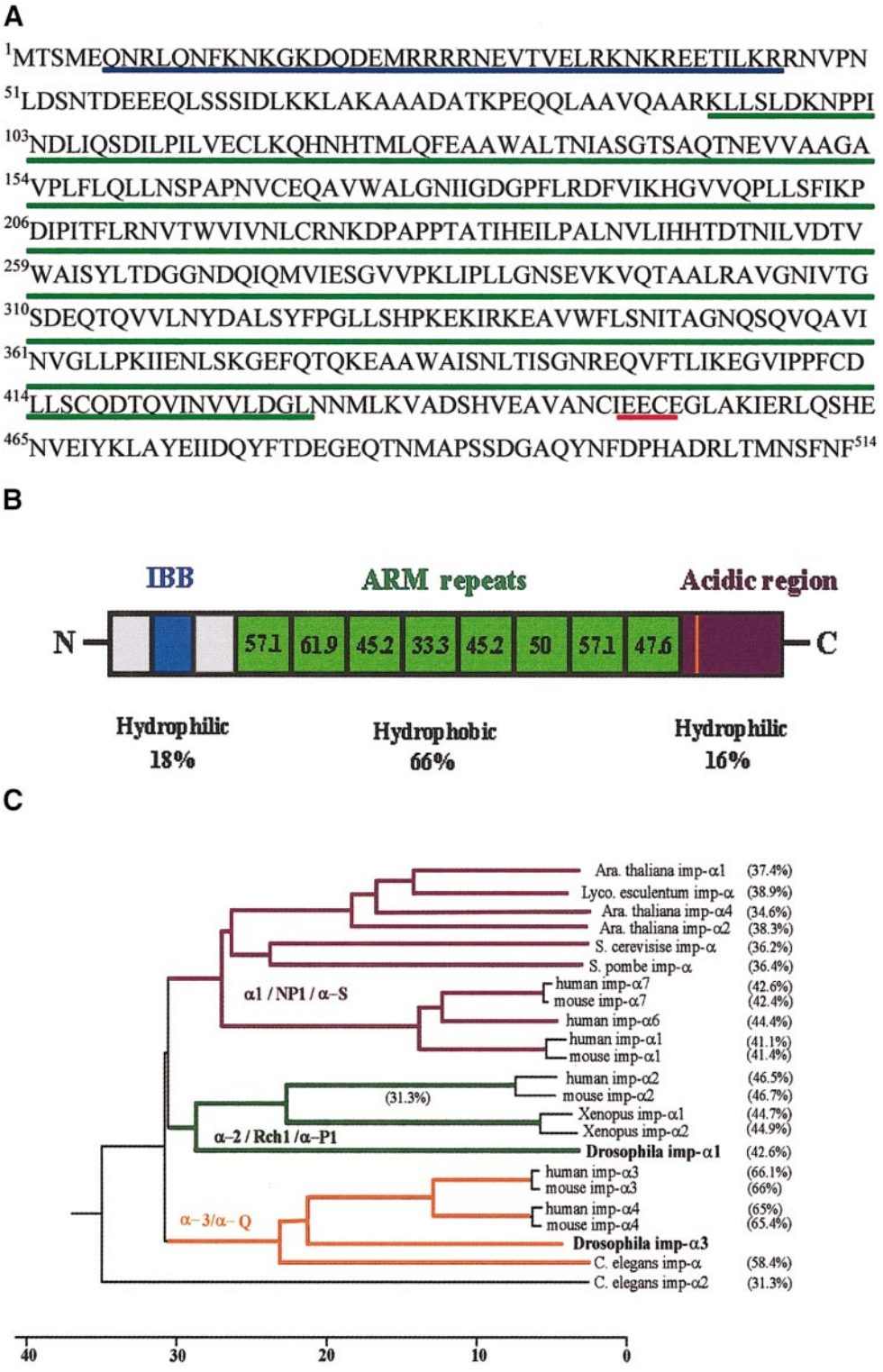


**FIG. 2.** Comparative analysis of the predicted structure of Importin-α3. (A) The predicted amino acid sequence of Importin-α3. The blue-underlined region represents the IBB domain and the green-underlined region contains the Armadillo repeats. The red-underlined region is a strongly acidic domain that might be involved in the binding of CAS (see Introduction). (B) Schematic representation of these regions of Importin-α3. In each Arm repeat of Importin-α3 the % homology is indicated compared to the original Arm repeat. (C) Relationship of importin-α homologues constructed using the CLUSTAL program. The % amino acid identity of Importin-α3 to the other members of the importin-α protein family is shown in parentheses using PAM250 residue weight.

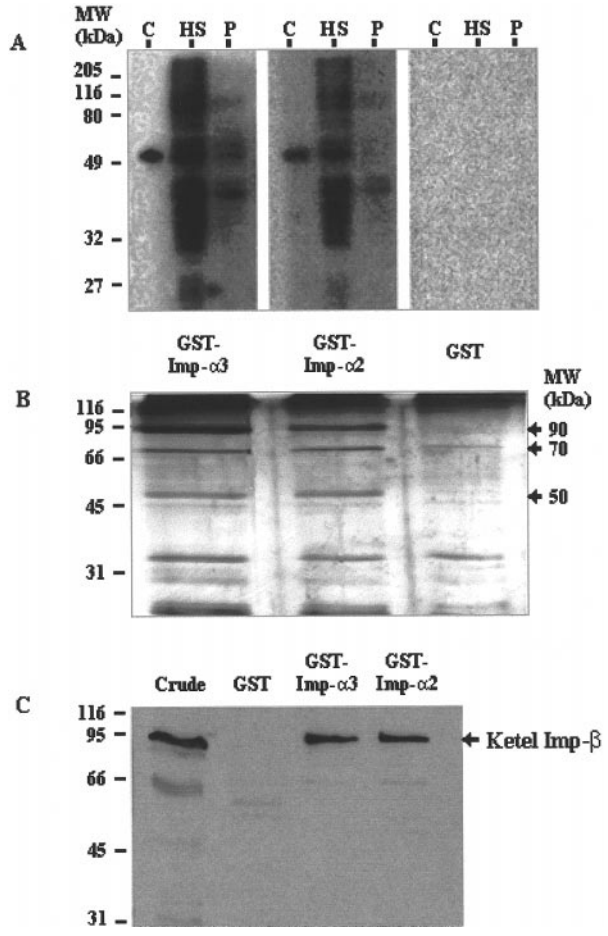


**FIG. 3.** Importin-α3 and Importin-α2 interact with similar subsets of proteins *in vitro*. (A) Electrophoretically separated protein fractions (cytoplasmic—C, high-salt nuclear—HS, and nuclear pellet—P, see Materials and Methods) of embryonic extracts were transferred to membranes and probed with $^{32}$P-labelled full-length GST–Importin-α3 and GST–Importin-α2. (B) Silver-stained SDS–PAGE analysis of protein fractions interacting with GST–Importin-α3 and GST–Importin-α2 immobilised on glutathione agarose. (C) Western blot of protein fractions as in B above probed with an antibody against the *Drosophila* importin-β homologue—Ketel.

### *Importin-α3 and Importin-α2 Show Different Patterns of Subcellular Localisation during the Division Cycles of Embryogenesis*

The *Drosophila* Importin-α2 protein has been previously shown to undergo dynamic relocalisation during the mitotic cycles of both syncytial and newly cellularised embryos, giving rise to its original name, Pendulin (Kussel and Frasch, 1995). Importin-α2 is predominantly cytoplasmic during interphase. At prophase it undergoes dramatic relocalisation into the nucleus and is redistributed into the cytoplasm at metaphase. In contrast, Importin-α3 tends to accumulate at the membrane of cleavage nuclei rather than inside them (Fig. 5). This is evident from the very earliest stages with the male and female pronuclei (Figs. 5A and 5B) and polar body nuclei (data not shown). Importin-α3 redistributes into the cytoplasm as soon as the nuclear membrane disassembles, marking the beginning of gonomeric or cleavage M phases (Figs. 5J and 5K), and reaccumulates at the membrane of daughter nuclei as early as telophase (Figs. 5D and 5E). The gonomeric division ensures the fusion of the parental chromosomes by means of a special spindle in which the male and female chromatids move to the poles in two separate groups (gonomery), mingling only when they reach the spindle poles at telophase (Huettner, 1924; Callaini and Riparbelli, 1996). However, the telophase-specific accumulation of Importin-α3 at the nuclear envelope is characteristic only of the gonomeric and first seven cleavage cycles. Following cortical migration (cleavage cycles 7–9), Importin-α3 is first seen at the nuclear membranes right at the beginning of the interphase of the next cleavage cycle and this localisation persists throughout the entire interphase (Figs. 5G and 5H). The blastoderm cellularisation takes place during the prolonged interphase 14 and it is the first important morphogenetic event that requires zygotic transcription (Yasuda *et al.*, 1991; Foe *et al.*, 1993). Following cellularisation Importin-α2 continues to follow the same pattern of subcellular distribution as in the syncytium, it is predominantly cytoplasmic during interphase, and moves into the nucleus at prophase (Kussel and Frasch, 1995). In contrast, we found that Importin-α3 is predominantly nuclear in interphase cells (Figs. 6D–6I) and becomes redistributed throughout the cell during M phase (Fig. 6C). We also found by immunostaining that Importin-α3 was present in the nuclei of interphase cells in different tissues and organs of third-instar larvae, including brains, gut, fat body, and salivary glands (data not shown).

To confirm the relative association of the two importin-α isoforms with the nucleus and cytoplasm we carried out subcellular fractionation. Proteins not associated with chromatin or other nuclear structures can be extracted from the nucleus by puncturing the nuclear membrane with detergent and washing with a low-salt buffer. More tightly bound proteins can then be extracted with higher salt washes. Western analysis was carried out on such fractions and Importin-α3 was found in both cytoplasmic and nuclear wash fractions (Fig. 4F). A 250 mM NaCl wash of the nucleus extracted more Importin-α3 that was nuclear bound. Surprisingly a greater amount was extracted with a 2 M NaCl wash and even after this there was still some remaining in the pellet, indicating that Importin-α3 is associated very strongly with the nucleus. A similar analysis showed that the bulk of Importin-α2 is associated with cytoplasmic extracts (Fig. 4E). A small fraction of Importin-α2 did associate with the nuclear fractions and some of this also seems to be tightly associated. Interestingly, the nuclear fractions seem to contain only the slower migrating form of Importin-α2, previously shown to be a phosphorylated form of the protein (Török *et al.*, 1995).

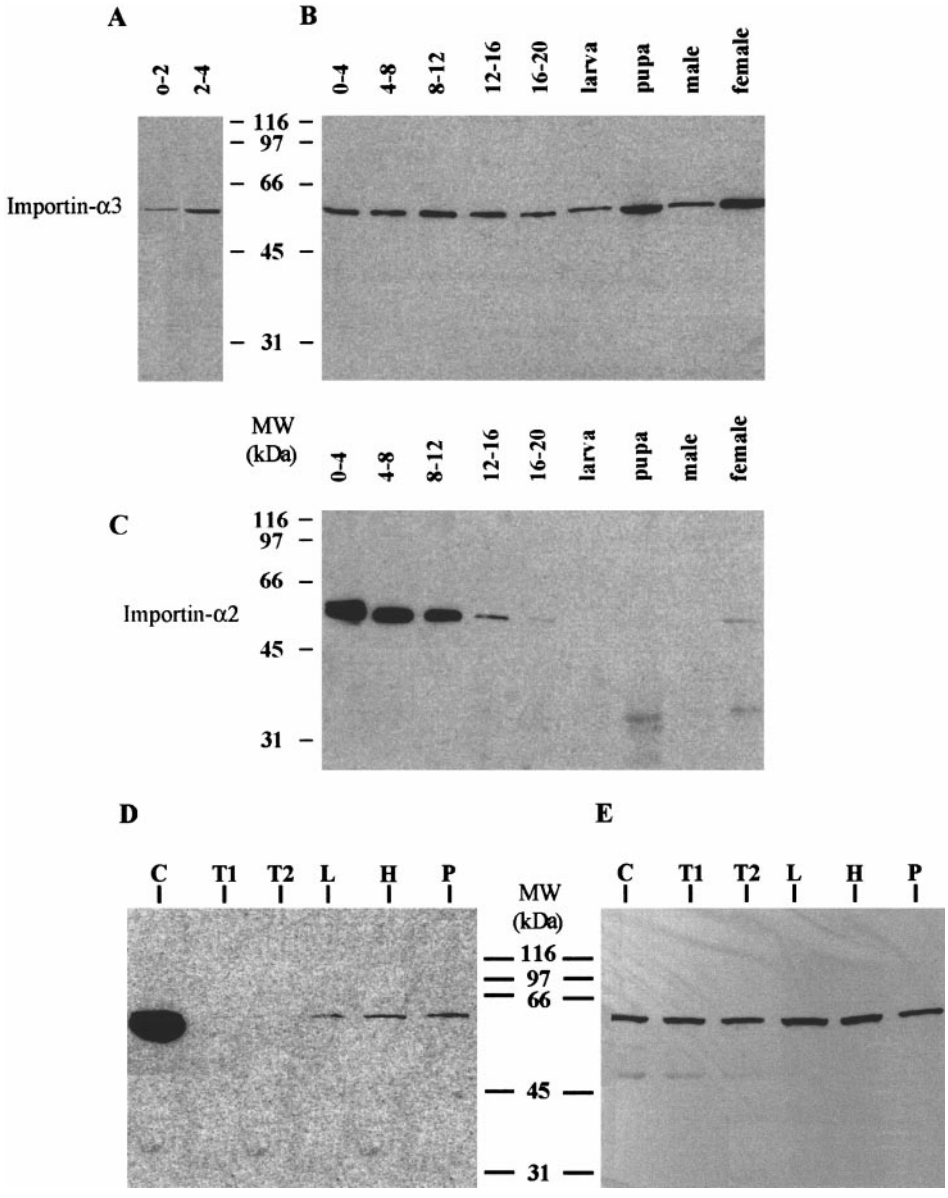


**FIG. 4.** Developmental Western analysis. Western blots to show the abundance of Importin-$\alpha$3 (A, B) and Importin-$\alpha$2 (C) throughout the individual wild-type developmental stages. Embryonic development is represented by the indicated age of embryos from 0 to 20 h of development. In all cases equal loading of samples was confirmed using an anti-tubulin antibody. (D, E) Western blot analysis of nuclear and cytoplasmic fractions from wild-type embryonic extracts probed with anti-Importin-$\alpha$2 (D) or anti-Importin-$\alpha$3 (E) antibodies. The fractions are C, cytoplasmic; T1, nucleoplasm wash 1; T2, nucleoplasm wash 2; L, low-salt wash (250 mM NaCl); H, high-salt wash (2 M NaCl); P, pellet. Only the slower migratory phosphorylated form of Importin-$\alpha$2 is found assembled with the nucleus (D—lanes L, H, and P), whereas both forms are found in the cytoplasm (D and E—lanes C).

We attempted to quantitate by Western blots the relative amounts of Importin-$\alpha$3 and Importin-$\alpha$2 per nucleus relative to known quantities of bacterially expressed proteins at stages of embryogenesis when expression of each protein appeared to be maximal. We calculate that the levels of Importin-$\alpha$3 rise to $2.5 \times 10^5$ molecules per nucleus in 4- to 8-h embryos, whereas there are $1.25 \times 10^8$ Importin-$\alpha$2 molecules per nucleus in 1-h embryos. It should be noted, however, that the majority of the Importin-$\alpha$2 is in the unphosphorylated form.

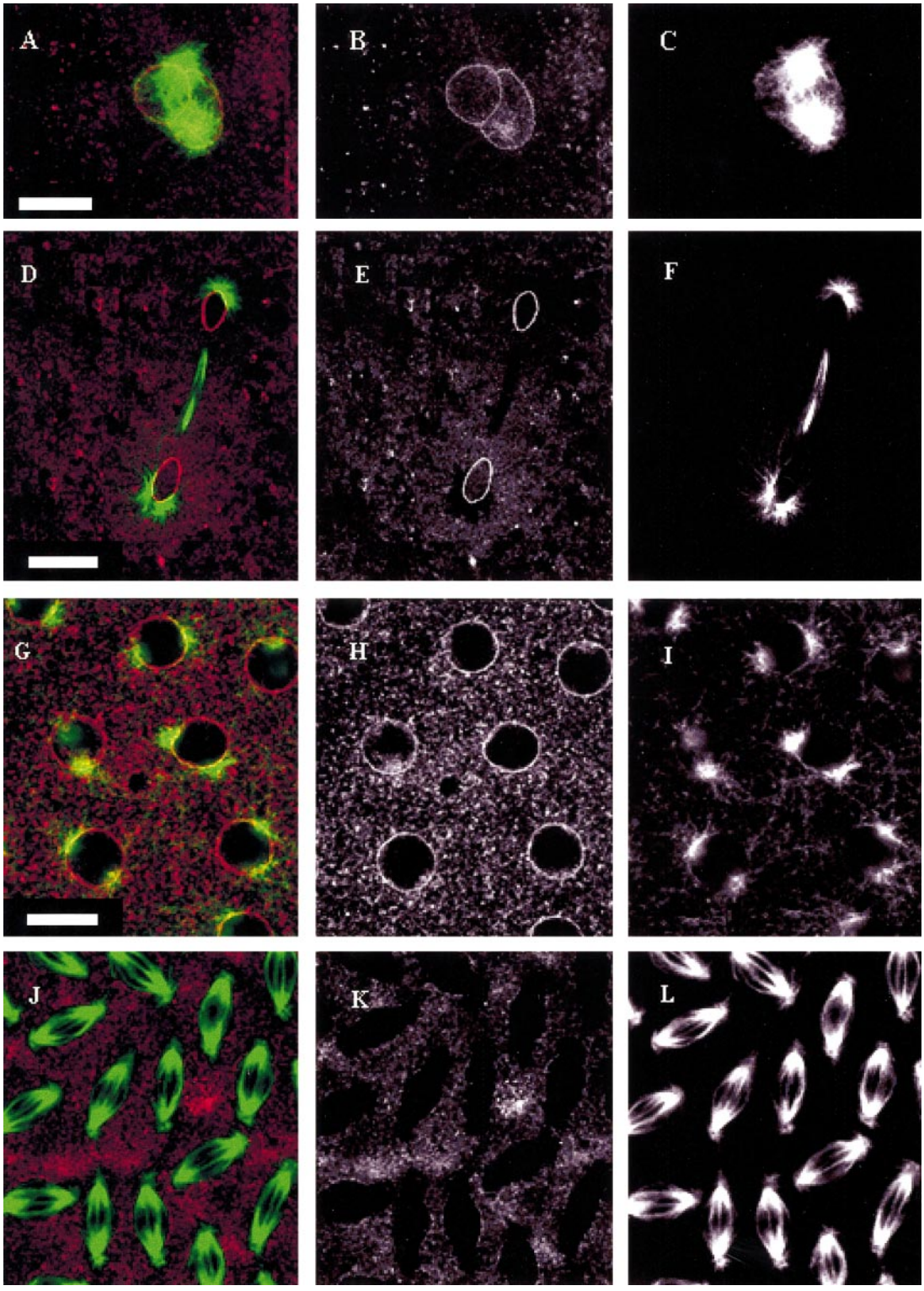


**FIG. 5.** Subcellular localisation of Importin-α3 in syncytial embryos. Confocal micrographs of wild-type embryos stained with anti-Importin-α3 (red in the merged image and middle column) and anti-α-tubulin (green in the merged image and right-hand column) antibodies. (A–C) At the pronuclear stage, Importin-α3 accumulates at the nuclear membrane of both female and male pronuclei at high levels, but its presence in the nuclei and cytoplasm is also evident. Bar, 10 μm. (D–F) The accumulation of Importin-α3 at the nuclear membranes is already seen in the late telophase of the gonomeric division. Bar, 10 μm. (G–L) A cleavage cycle 8 embryo showing the Importin-α3 accumulation at the nuclear membranes of cleavage nuclei during the S phase (G–I). The metaphase of cleavage cycle 8 (J–L) showing the Importin-α3 is exclusively cytoplasmic. Bar, 10 μm.

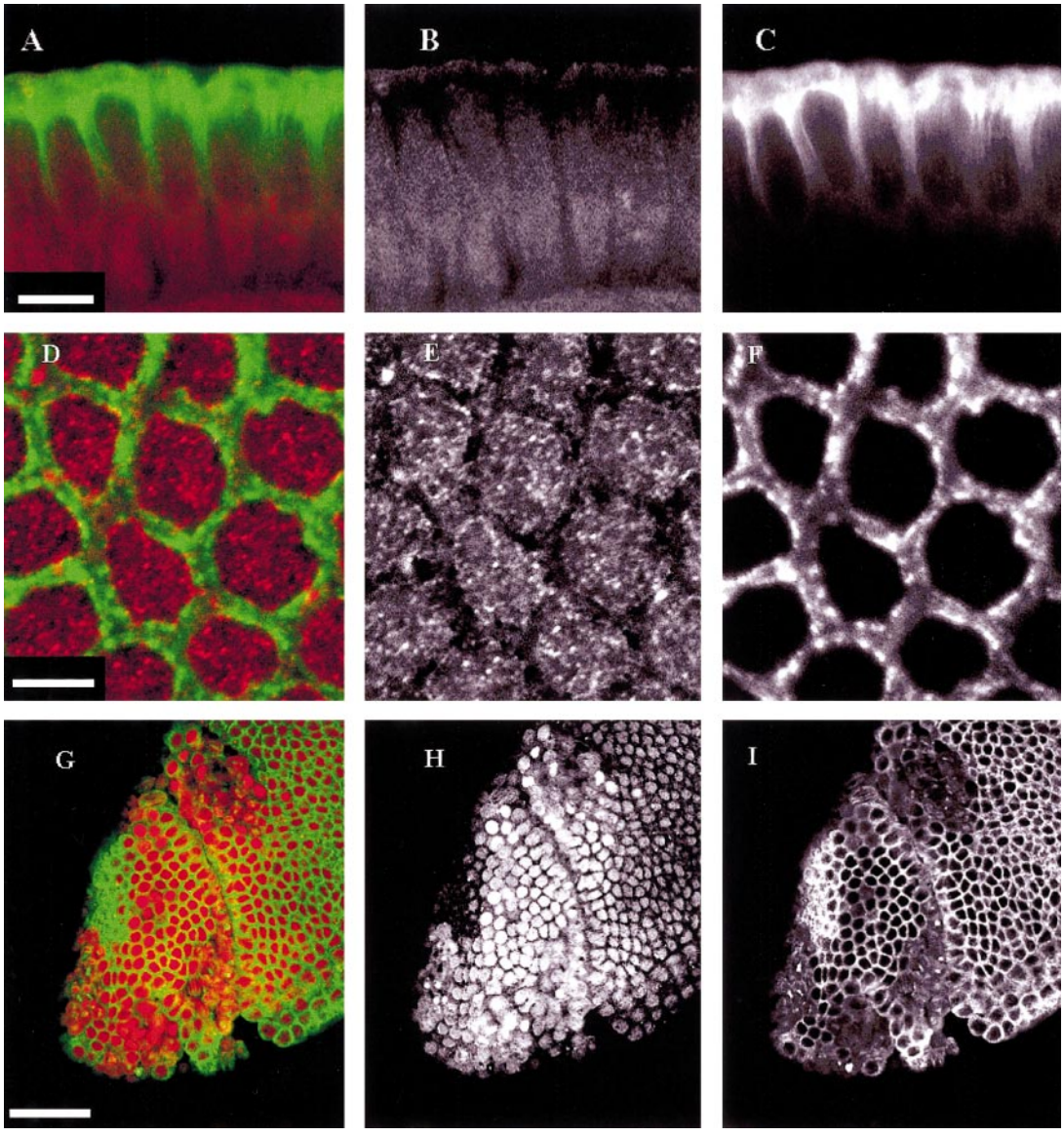


**FIG. 6.** Subcellular localisation of Importin-$\alpha$3 in late cellularised embryos. Confocal micrographs of wild-type embryos stained with anti-Importin-$\alpha$3 (red in the merged image and middle column) and anti-$\alpha$-tubulin (green in the merged image and right-hand column) antibodies. Longitudinal (A–C) and horizontal (D–F) sections through the same cellular blastoderm stage embryo to show the accumulation of Importin-$\alpha$3 in nuclei during interphase. Bars, 10 $\mu$m. (G–I) The cephalic region of a gastrulating embryo in which interphase nuclei contain elevated levels of Importin-$\alpha$3. Bar, 50 $\mu$m.

### *Expression of Importin-α3 during Oogenesis Reflects the Demand for Nuclear Import*

Oogenesis in *Drosophila* provides the oocyte with the dowry of maternal proteins that enable the development of syncytial embryos. This is achieved over a 2- to 3-day period (corresponding to egg chamber stages 2–10), when nurse cells undergo 10–12 endocycles, and by an intensive biosynthetic activity (stages 7–10) produces the majority of the future egg contents (Dey and Spradling, 1999). In contrast, the oocyte nucleus transcribes actively in germarial cysts (stage 2) and becomes repressed shortly after the egg chambers leave the germarium (stage 3), and its chromosomes condense into the karyosome, which it is presumed to be transcriptionally silent. Moreover, the egg chamber is initially surrounded by a monolayer of follicular cells that obtain lower levels of polyploidy but also undergo the amplification of subsets of genes to facilitate the biosynthesis of the chorion. We have found dramatic changes in the

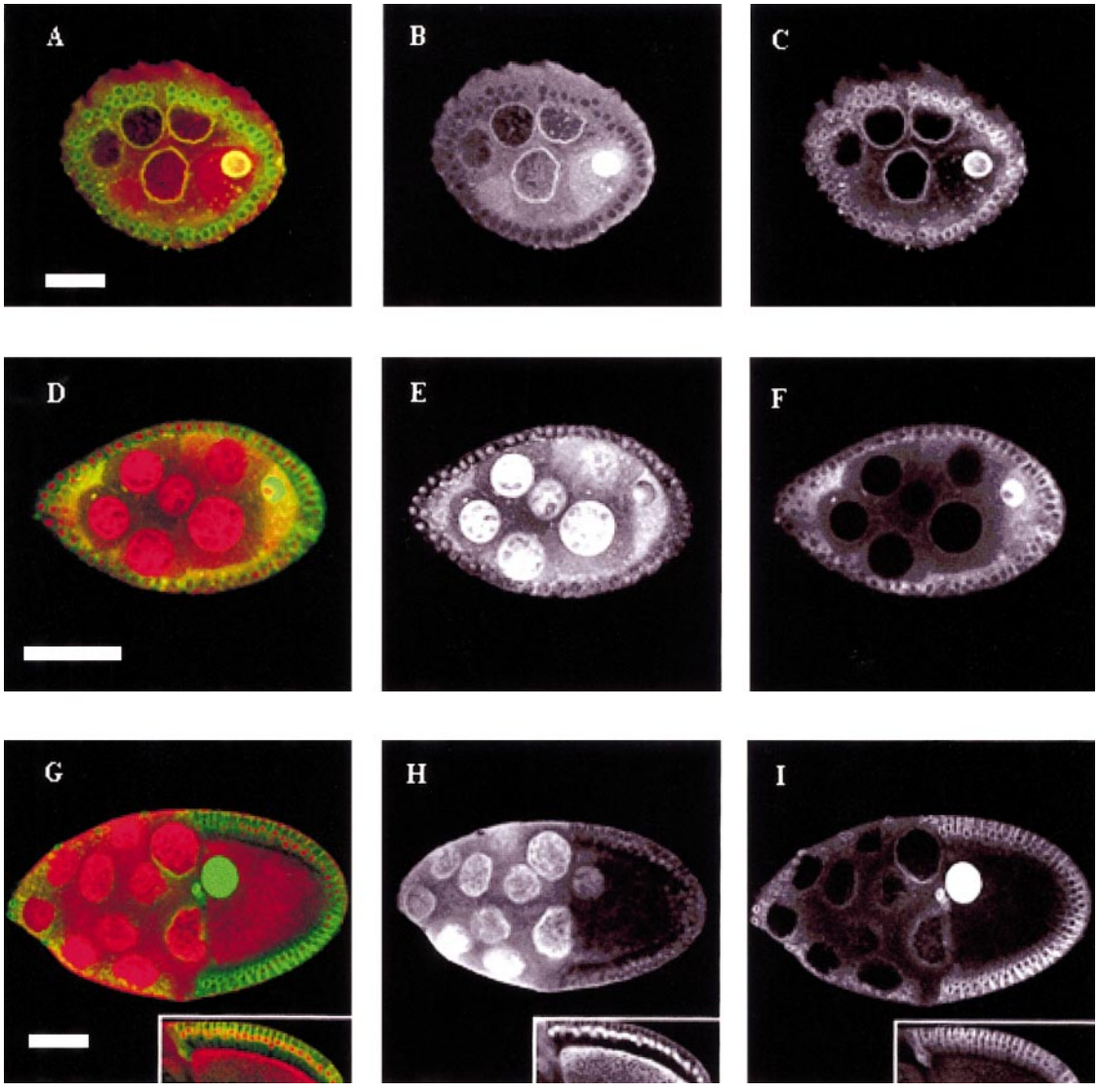


**FIG. 7.** Subcellular localisation of Importin-α3 during oogenesis. Confocal micrographs of wild-type egg chambers stained with anti-Importin-α3 (red in merged image and middle column) and anti-lamin (green in merged image and right-hand column) antibodies. (A–C) At stage 5 of oogenesis Importin-α3 is uniformly distributed in the cytoplasm of both nurse cells and oocyte. The oocyte nucleus and cytoplasm contain elevated levels of Importin-α3 compared to the nurse cells. Importin-α3 is present at a low level in nurse cell nuclei, while in their nuclear membranes it accumulates at a significant level. Importin-α3 is not seen in either nuclei or nuclear membranes of follicular cells. Bar, 25 μm. (D–F) At stage 7 of oogenesis both nurse cell and follicular cell nuclei show increased levels of Importin-α3, while in the oocyte nucleus Importin-α3 is present at a lower level than in stage 4 of oogenesis. Bar, 50 μm. (G–I) An egg chamber at stage 10 of oogenesis sectioned in a focal plane close to its surface (insets show a central focal plane). Importin-α3 is present at high levels in nurse cell nuclei. It also accumulates in the cortical region of the oocyte cytoplasm and in follicular cell nuclei, while in the oocyte nucleus it is present at a very low level. Bar, 50 μm.

subcellular localisation of Importin-α3 in specific cell types during oogenesis (Fig. 7). In stages 2–6 of oogenesis, Importin-α3 accumulates at the membrane of both nurse cell and oocyte nuclei (Figs. 7A and 7B). There appears to be little Importin-α3 within the nurse cell nuclei, whereas it is abundant in the oocyte nucleus at these stages. During stages 7–10, nurse cell and follicular cell nuclei accumulate high concentrations of Importin-α3, while levels in the oocyte nucleus decline (Figs. 7E and 7H and inset in 7H). Subsequently during stages 10–12, Importin-α3 accumulates in the cortical cytoplasm of the oocyte (insets in Figs. 7G and 7H). During stage 13 (corresponding to promet-

aphase of meiosis I) and stage 14 (corresponding to metaphase of meiosis I), Importin-α3 persists in the cortical cytoplasm of matured eggs (data not shown). The follicular cell nuclei show little Importin-α3 during oogenetic stages 2–6 (Figs. 7A and 7B), but this increases markedly throughout stages 7–12 (Figs. 7D and 7E and inset in 7H), declining with the degeneration of the follicular cells from stage 13 (data not shown).

### *The imp-α3 Mutants Are Poorly Viable and Female Sterile*

In order to gain some insight into the functions of Importin-α3 we sought to identify mutations in its gene. We identified a P-lacW mutant line from the collection of Deák *et al.* (1997) in which the transposon was inserted into the cytological interval to which we had mapped the *imp-α3* gene by *in situ* hybridisation. Rescue of the P-lacW flanking genomic sequences from this line indicated that the P-lacW element was inserted 550 bp upstream of the first ATG codon in the *imp-α3* open reading frame (Fig. 1). We therefore tentatively designated this mutant allele *imp-α3*$^1$. Homozygous *imp-α3*$^1$/*imp-α3*$^1$ and hemizygous *imp-α3*$^1$/*Df(3R)by62* individuals show pharate adult lethality and female sterility. The cytological interval in which the mutation lies was narrowed to 85E10–F16 since the *imp-α3*$^1$/*Df(3R)by10* individuals were viable and fertile, indicating that the gene lies in the interval 85E10–F16, consistent with the insertion site of the P-lacW element (Fig. 1). To demonstrate that the P-lacW element was associated with the partially lethal mutation we placed the *imp-α3*$^1$ mutant line under dysgenic conditions and selected revertants in which the P-lacW element had been remobilised. Sixty-two revertants were isolated that were viable and female fertile when homozygous and had $w^-$ eyes in a $w^-$ background, indicating the complete reversion of the *imp-α3*$^1$-associated mutant phenotypes and the precise excision of the P-lacW element. These complete revertants crossed back to the original *imp-α3*$^1$ allele or the *Df(3R)by62* gave viable and fertile offspring, strongly suggesting that the P-lacW insertion is responsible for the *imp-α3*$^1$-associated mutant phenotype. These reversion tests also generated 10 other partial revertants whose mutant phenotypes can be accounted for by imprecise excision and/or local hopping of the P-lacW element. The partial revertants gave viable and fertile offspring over *Df(3L)by62,* while under homozygous conditions they were lethal, indicating that remobilisation had resulted in mutations in other complementation groups or genes.

In order to define the *imp-α3*$^1$ mutant allele, we undertook germ-line transformation experiments to determine whether the cloned *imp-α3* gene would rescue the mutant phenotypes. We screened the European *Drosophila* Genome Project cosmid library using the P-lacW flanking chromosomal sequences and cDNA clones. We identified cosmids carrying the gene from which we selected a 9-kb *Eco*RI restriction fragment for insertion into the P-element germline transformation vector (Fig. 1). This fragment of genomic DNA (pR4, Fig. 1) was able to rescue all mutant phenotypes associated with the *imp-α3*$^1$ allele. As a negative control we used a variant of the germ-line transformation construct in which most of the *imp-α3* gene was deleted (pRD4, Fig.1) and which was unable to rescue the mutant phenotypes.

We have been unable to pinpoint a specific cause of pharate adult lethality in homozygous and hemizygous *imp-α3*$^1$ mutants. Examination of orcein-stained preparations of brains from mature third-instar larvae failed to reveal any mitotic defects and in fact the mitotic index was identical to that seen in wild-type brains (data not shown), suggesting there are no generalised defects in cell proliferation.

The *imp-α3*$^1$ allele seems to be hypomorphic since the Western blot of ovarian total protein extracts shows a reduced level of Importin-α3 in *imp-α3*$^1$/*imp-α3*$^1$ females compared to wild-type (Fig. 4C). The sterile homozygous *imp-α3*$^1$ mutant females have defective ovaries that show fewer ovarioles (4–10 per ovary) and degenerated egg chambers (Fig. 8). Whereas some nuclei within the germarium contain Importin-α3 (region a in Figs. 8A and 8B), in contrast to the wild-type stage 7–10 egg chambers, neither nurse cell nor follicular cell nuclei accumulate detectable amounts of Importin-α3 (Figs. 8C and 8D, compare with Figs. 7D and 7E). Typically the egg chambers in such mutant ovaries degenerate at stage 7–10 of oogenesis although in some ovaries one or two eggs may reach maturity, exhibiting normal chorional appendages (data not shown), but they are never released from the ovaries. Stage 7–10 of oogenesis is a time at which the nurse cells should have reached their maximum ploidy in readiness to undertake the biosynthetic activity necessary to provide the oocyte with its maternal dowry.

## DISCUSSION

### *Isolation of importin-α Homologues from Drosophila melanogaster*

In this study we have identified two karyopherin-α or importin-α homologues from *Drosophila* as proteins that interacted in a yeast two-hybrid screen with the large subunit of DNA polymerase-α (p180). This is perhaps not surprising since *Drosophila* p180 shows homology to a region in the *Schizosaccharomyces pombe* DNA polymerase-α, which has been shown to contain a functional bipartite NLS (Bouvier and Baldacci, 1995). It is highly likely that the two *importin-α* genes of *Drosophila* were detected in our screen through the interaction of their proteins with the NLS of the bait protein.

It has been known for some time that mammals (mouse and human) have several importin-α homologues, which fall into three subfamilies. The isolation of a second importin-α from *Drosophila* now suggests that this phenomenon is widespread among multicellular organisms and

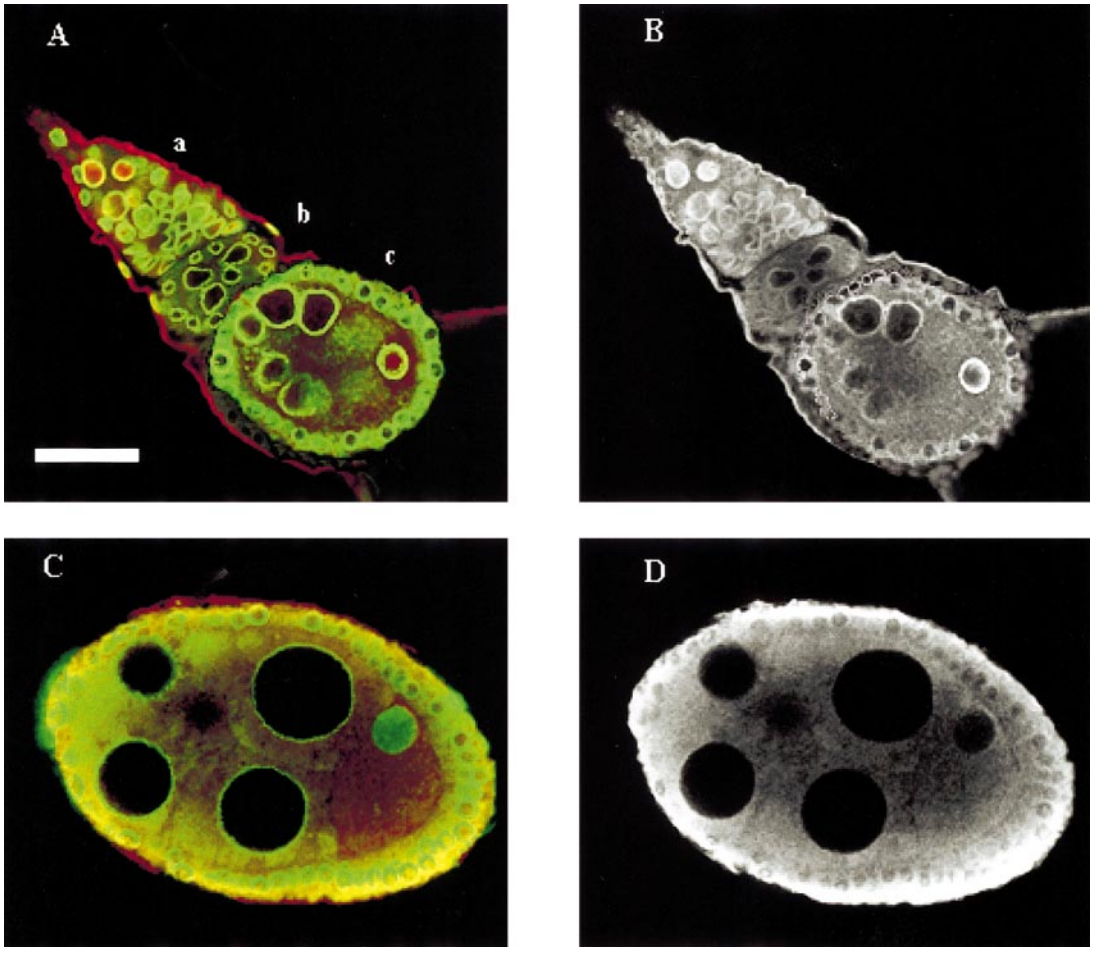


**FIG. 8.** Subcellular localisation of Importin-α3 in *imp-α3*$^{1}$/*imp-α3*$^{1}$ ovaries. Confocal micrographs of mutant egg chambers stained with anti-Importin-α3 (red in the merged image and right-hand column) and anti-lamin (green in the merged image) antibodies. (A, B) Mutant ovariole showing some Importin-α3 staining in occasional nuclei in the germarium (a) and egg chamber at stage 4 (b). In stage 6 egg chamber (c) it is difficult to detect Importin-α3 in either nurse cell or oocyte nuclei. Bar, 25 μm. (C, D) Mutant, stage 9 egg chamber showing no accumulation of Importin-α3 in the nurse cell and follicular cell nuclei. Oogenesis proceeds no further than this stage in the majority of ovarioles. Bar, 50 μm.

not just specific to mammals. Analysis of the amino acid sequence of the two *Drosophila* importin-α homologues has allowed us to assign each to a particular subfamily. The previously identified Oho31/Pendulin shows highest homology to the subfamily α2, and we therefore suggest that this should be renamed Importin-α2. The newly identified *Drosophila* importin-α homolog, however, is much closer to the α3 subfamily, and we have therefore named this protein Importin-α3. The fact that the two *Drosophila* homologues are more closely related to homologues from other species than each other suggests an overriding functional requirement for the regions that are conserved.

The region of the protein which is most conserved is the region containing the eight Arm repeats. Arm repeats were originally identified in the Armadillo protein from *Drosophila* (Riggleman *et al.*, 1989), but have since also been found in a number of proteins with a wide variety of functions such as β-catenin, plakoglobin, the tumour suppressor protein APC (which interacts with β-catenin), and the GDP/GTP exchange factor smgGDS (Peifer *et al.*, 1992). It has been suggested that Arm repeats mediate strong protein–protein interactions. In addition, individual or small groups of Arm repeats may have particular functions, as studies on Armadillo suggest that each individual repeat is able to mediate weak protein–protein interactions (Peifer and Wieschaus, 1995). The Arm motifs in the importin family have been shown to mediate interactions with the NLS (Conti *et al.*, 1998). Certain of the corresponding Arm motifs in Importin-α2 and Importin-α3 show high identity, which may suggest that some repeats are more important than others for the interaction to occur. There does seem to be some redundancy of function in the Arm repeats since

the initial Importin-α3 clone, missing the N-terminal portion of the protein up to the third Arm motif, was still apparently capable of NLS interactions (data not shown). This is also consistent with results from Prieve *et al.* (1996), who showed that a truncated form of human importin-α1 (hSrp1) missing the first two Arm motifs could still interact with an NLS-protein.

### *Analysis of Cellular Functions of Importin-α Isoforms*

The presence of conserved multiple importin-α isoforms in many species suggests that each of them may have a specific function. It is possible that each homologue is responsible for the transport of a unique subset of proteins in a controlled spatial and temporal pattern as was suggested for human (Köhler *et al.*, 1997) and mouse (Tsuji *et al.*, 1997; Kamei *et al.*, 1999). In this case we might expect to see each importin-α isoform showing different substrate specificities. Alternatively, it is possible that some of the isoforms are functionally redundant by featuring overlapping substrate specificities. In this case the importin-α isoforms would be expected to have similar substrate affinities, and any specificity in the system would be introduced by the binding properties of individual substrates and the levels of the proteins that are available. The different expression levels and/or subcellular distributions of the importin-α isoforms during development could therefore represent a requirement for the nuclear import of specific proteins and/or levels of generally increased nuclear protein import. Therefore the phenotypes of mutants in each case should also reflect stages in development or cell cycle at which there is a specific requirement for each importin-α isoform. In this case the phenotype of the mutant might also help to define the role of the importin-α isoform specific to a particular developmental or cell cycle stage, whereas a less defined developmental arrest would suggest pleiotropic function.

### *Importin-α3 and Importin-α2 Interact with the Same Range of Proteins in Vitro*

Our molecular and biochemical studies suggest that the protein interactions made by Importin-α3 and Importin-α2 are very similar under *in vitro* conditions. The GST-tagged Importin-α3 and Importin-α2 bound similar profiles of proteins both in solution and immobilised following electroblotting. Furthermore similar patterns of potentially interacting proteins were identified within immunoprecipitates. A particularly large number of interacting proteins were found in washes of high-salt nuclear preparations. It is likely that the more tightly interacting bands represent components of the NLS-dependent nuclear transport machinery. This is confirmed by the identification of the 90-kDa band as the *Drosophila* homologue of the importin-β protein. Identification of the 50- and 70-kDa bands has not been possible since *Drosophila* homologues of other components of the NLS-dependent nuclear transport machinery have not yet been reported. The weaker interacting bands may be additional components of the transport machinery or transport substrates.

Although our results argue that the two importin-α isoforms are very similar in terms of the contacts that they make *in vitro*, we remain cautious as to whether they reflect interactions *in vivo* since the proteins are perhaps forced into unusual interactions due to the nonphysiological conditions used. Moreover, we might be seeing only a subset of the interactions taking place, for example, with the transport machinery and are therefore missing crucial differences between substrates.

It is also possible that phosphorylation might play some role in controlling the binding affinities of the two importin-α isoforms of *Drosophila*. The protein used for our binding studies was not phosphorylated since it is of bacterial origin, but it may become phosphorylated during incubation with the embryonic extracts. However, we do not know whether this occurs or the extent to which there could be a preference for nonphosphorylated forms by the interacting proteins. A phosphorylated form of Importin-α2 can be detected in early embryos and in ovaries (Török *et al.*, 1995). However, we have not been able to detect a phosphorylated form of Importin-α3 at any stage of development. Since phosphorylated Importin-α2 is the only form seen in the nucleus it is tempting to speculate that phosphorylation is necessary for activity. This could be a means of regulating the utilisation of large maternal input of Importin-α2 to early embryos. It is also possible that phosphorylation could control substrate recognition or the prophase-specific interaction of Importin-α2 with the NLS-dependent nuclear transport machinery. One intriguing observation with respect to this alternative is that Importin-α2 has a potential Cdc2 phosphorylation site adjacent to the IBB domain. If it were necessary to phosphorylate this site in order to get interaction with Importin-β it is easy to see how this would help to control the activity of the protein. Importin-α3 in contrast has no such phosphorylation site, but has instead a run of negatively charged residues at a similar location, which alternatively may provide the cell cycle control of its function.

### *Importin-α3 and Importin-α2 Show Different Developmental Profiles*

Importin-α3 and Importin-α2 show dramatically different timing of expression during the life cycle of *Drosophila*. The appearance of Importin-α2 in the embryos coincides with the time of intense mitotic activity, which presumably imposes specific demands upon traffic to and from the nucleus. On the other hand levels of Importin-α3 in the syncytial embryos are very low and do not increase until after cellularisation, following which they appear to remain apparently at a constant level throughout development. The simplest rationalisation of the expression patterns is that Importin-α3 functions as the main "housekeeping"

importin-α with Importin-α2 playing an additional or more specialised role during embryogenesis consistent with its prophase-specific nuclear accumulation (Kussel and Frasch, 1995; Török *et al.*, 1995). In contrast to the predominant distribution of Importin-α2 in the interphase cytoplasm, Importin-α3 is principally nuclear in interphase although its intensity of nuclear staining also increases in prophase to become more dispersed at metaphase. However, it is intriguing that although Importin-α3 is present mainly within the nucleus at most developmental stages, it is distinctly associated with the nuclear envelope in the early embryos before its levels reach their maximum and also in the endoreduplicating nurse cells before they reach their maximal ploidy during oogenesis. The significance of this is not clear, but the association with the nuclear envelope may anticipate a requirement for a higher amount of Importin-α3 prior to it filling the entire nucleus.

### *Importin-α3 Is Required to Complete Pupal Development and Oogenesis*

The differences that we have observed in the developmental and subcellular localisation profiles of Importin-α3 and Importin-α2 indicate that the two proteins are not redundant in the context of normal development. However, whether this reflects differential control of expression of proteins or particular substrate specificity for each protein is uncertain. It will require a detailed analysis of the cellular functions of both proteins at critical stages of development. The pleiotropy of *imp-α3*$^{1}$ mutant phenotypes suggests that the Importin-α3 has a general house keeping function and that the developmental processes affected reflect the point at which the levels of the protein become rate limiting. We were unable to pinpoint the precise nature of these defects in the pupal stages. However, oogenesis is interrupted specifically at stage 7–10 a time at which large amounts of Importin-α3 would normally be present in the wild-type nurse cell nuclei. It therefore seems likely that Importin-α3 is required at this stage for transport of NLS proteins into the nurse cell nuclei. This precedes a crucial transition in oogenesis when at stage 10, the nurse cell contents are dramatically transported into the oocyte cytoplasm (Spradling, 1993).

## ACKNOWLEDGMENTS

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